Multicenter Immunohistochemical ALK-Testing of Non-Small-Cell Lung Cancer Shows High Concordance after Harmonization of Techniques and Interpretation Criteria

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Abstract: INTRODUCTION Detection of anaplastic lymphoma kinase (ALK)-gene rearrangements in non-small-cell lung cancer (NSCLC) is mainly performed by fluorescence in-situ hybridization (FISH). The question was raised if FISH might be replaced by immunohistochemistry (IHC) in a reliable and reproducible manner across different laboratories. METHODS After calibration of the staining instruments and training of the observers to binary interpretation (positive versus negative), 15 NSCLC were independently tested for ALK protein expression by IHC only in a multicenter setting (16 institutes). Each laboratory utilized the VENTANA ALK-D5F3 IHC assay. As demonstrated by FISH the samples displayed unequivocal ALK break-positivity (6×) and negativity (7×), as well as ALK positive-"borderline" character (2×), which is challenging for FISH diagnosis and thus was RT-PCR-confirmed. RESULTS All seven ALK FISH-negative cases were homogenously scored as ALK-IHC negative. All 16 participants scored the two ALK positive-"borderline" samples as unequivocally positive according to their protein expression. Concordant IHC interpretation was also noticed in four of six unequivocal ALK break positive cases. In two of six some observers described a weak/heterogeneous ALK-IHC staining. This would have resulted in a subsequent ALK-testing (FISH/PCR) in a routine diagnostic setting. CONCLUSIONS This so-called "ALK-Harmonization-Study" shows for the first time that predictive semiquantitative IHC reveals reliable and reproducible results across several labs when methodology and interpretation are strictly defined and the pathologists are uniquely trained. The application of validated ALK IHC assays and its comparison to ALK-FISH is highly needed in future clinical trials. This might answer the question if ALK-IHC cannot only serve as a prescreening tool, but as a stand-alone test at least in cases displaying an unequivocally staining pattern as well as an alternative predictive test in samples with reduced FISH interpretability.

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Introduction: Detection of anaplastic lymphoma kinase (ALK)-gene rearrangements in non–small-cell lung cancer (NSCLC) is mainly performed by fluorescence in-situ hybridization (FISH). The question was raised if FISH might be replaced by immunohistochemistry (IHC) in a reliable and reproducible manner across different laboratories.

Methods: After calibration of the staining instruments and training of the observers to binary interpretation (positive versus negative), 15 NSCLC were independently tested for ALK protein expression by IHC only in a multicenter setting (16 institutes). Each laboratory utilized the VENTANA ALK-D5F3 IHC assay. As demonstrated by FISH the samples displayed unequivocal ALK break-positivity (6×) and negativity (7×), as well as ALK positive-"borderline" character (2×), which is challenging for FISH diagnosis and thus was RT-PCR-confirmed.

Results: All seven ALK FISH-negative cases were homogenously scored as ALK-IHC negative. All 16 participants scored the two ALK positive-"borderline" samples as unequivocally positive according to their protein expression. Concordant IHC interpretation was also noticed in four of six unequivocal ALK break positive cases. In two of six some observers described a weak/heterogeneous ALK-IHC staining. This would have resulted in a subsequent ALK-testing (FISH/PCR) in a routine diagnostic setting.

Conclusions: This so-called "ALK-Harmonization-Study" shows for the first time that predictive semiquantitative IHC reveals reliable and reproducible results across several labs when methodology and interpretation are strictly defined and the pathologists are uniquely trained. The application of validated ALK IHC assays and its comparison to ALK-FISH is highly needed in future clinical trials. This might answer the question if ALK-IHC cannot only serve as a prescreening tool, but as a stand-alone test at least in cases displaying an unequivocally staining pattern as well as an alternative predictive test in samples with reduced FISH interpretability.

Key Words: Non–small-cell lung cancer, Anaplastic lymphoma kinase gene rearrangement, Immunohistochemistry, Harmonization, Multicenter-validation.

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Rearrangements in the anaplastic lymphoma kinase (ALK) gene are detectable in approximately 2 to 7%[1,2] of non–small-cell lung cancers (NSCLC). A paracentric inversion within the ALK-gene of chromosome 2 leads to a fusion with the EML4-gene or rarely with other partners (translocation: KIF5B, TFG, and KLC1)[1–3] and results in an expression of the oncogenic ALK-protein, providing a target for ALK-tyrosine kinase inhibitors (TKI).[4–6] ALK rearrangements mostly occur in

KRAS and EGFR wild type adenocarcinomas and preferentially arise in young and nonsmoking patients.[7–9] As the identification of ALK activity is crucial for the prescription of ALK-TKIs, a reliable diagnostic detection is of utmost importance. The studies leading to the approval of this therapeutic regimen were based on fluorescence in-situ hybridization (FISH), the current diagnostic gold standard.[2,4–6] However, FISH is expensive, time-consuming, and requires special equipment. Still, the detection of ALK rearrangements may not only fail due to technical (e.g., auto-fluorescence, overdigestion) and material-based reasons (e.g., low number of tumor cells), but also can be complex and challenging, as the most common alteration, the intrachromosomal inversion, may lead to subtle separations of the 5′and 3′signals. This has led to false-positive and false-negative interpretations.[10–15] As recent studies based on immunohistochemistry (IHC) showed promising results, the detection of the ALK protein was suggested as a further diagnostic option.[15–21] However, to perform ALK testing with robust reproducibility and reliability across different laboratories a careful validation is required. This necessity was addressed in the here presented "ALK-Harmonization-Study": after harmonization of the technical procedure and webinar-based ALK-IHC interpretation training of all observers (16 institutes of pathology), the participants had to perform ALK-testing (negative versus positive) of 15 well-characterized NSCLC samples by IHC only.

MATERIAL AND METHODS

Tumor Sample Selection and Pretesting by FISH

Fifteen well-characterized NSCLC samples (12× adenocarcinoma, 1× adenosquamous carcinoma, 1× squamous cell carcinoma, and 1× cell line [H2228]; Table 1) had been selected out of a previously described cohort (retrospective) of lung cancer tissues.[12] FISH pretesting was performed on whole slide tissue sections. Four-micrometer thick sections were cut, mounted on SuperFrost +/+ slides, and deparaffinized. A commercially available break-apart dual color probe for the ALK gene (Vysis LSI ALK Dual Color; Abbott Molecular, Abbott Park, IL) was used in accordance to the manufacturer's instructions. The 5′ALK probe was labeled with SpectrumGreen and the 3′ALK probe with SpectrumOrange. One hundred non-overlapping cells with hybridization signals were examined for each case with a fluorescence microscope (Axio Imager Z1, Zeiss, Jena, Germany) at a 63× oil immersion objective. ALK-FISH was considered positive if at least 15% of 100 analyzed tumor cells showed splitting of the fluorescent probes flanking the ALK-gene or loss of the 5′ signal, as described elsewhere.[2,7,12,15–17,19,22] Seven samples were unequivocally ALK break-negative and displayed a maximum of 3% split signals (SS). One of these seven samples (a squamous cell carcinoma) was included because of its unusual; however, ALK-break negative FISH pattern with single green signals in approximately 70% of the tumor cells. Six samples were unequivocally ALK break-positive and displayed SS and/or single red signals (SRS) in at least 40% of the tumor cells (mainly one alteration per nucleus). One of these six samples was currently reported as "new" EML4-ALK variant[23] that displays a fusion of Exon 6 (EML4) and Exon 19 (ALK). The remaining two cases were classified as ALK positive-"borderline" NSCLC, as showing a low percentage of ALK break-positive cells (around the cut-off of 15%) by FISH analysis. Both had been confirmed as EML4-ALK variant 1 and 3a/b by RT-PCR and subsequent sequencing of the breakpoints (no therapy response data were available in these samples).

Tissue Microarray (TMA) Construction and Retesting at Leading Sites

Two TMAs consisting of the above-described 15 NSCLC samples were constructed (three cores for each case, 1-mm diameter per core, resulting in a total of 45 cores). TMA-1

TABLE 1. Fifteen NSCLC Samples and Their ALK Status (Positive or Negative) by Means of IHC and FISH as Well as Their RT-PCR Products (Variants 1, 2, 3a/b, Atypical Variant, 3′ALK-Transcript)

Case	ALK-IHC	ALK-FISH	RT-PCR	Comment
1	Pos.	SS, SRS (~69%)	Variant 3a/b	
2	Neg.	Neg. (<3%)	Neg.	
3	Pos.	Atypical SS/SRS (~80%)	3′ALK-transcript	"Mickey-mouse" "Loss of red" (not a Variant 1,2,3)
4	Neg.	Neg. (<3%)	Neg.	Pitfall in IHC (macrophages)
5	Neg.	Neg. (<3%)	Neg.	
6	Pos.	SS, SRS (~15–20%)	Variant 1	"Borderline case"; ADC+SCC
7	Neg.	Neg. (<3%)	Neg.	
8	Pos.	SRS (~80%)	3′ALK-transcript	Not a Variant 1,2,3
9	Neg.	Neg. (<3%)	Neg.	Pitfall in IHC (necrosis); SGS in FISH; SCC
10	Pos.	SS, SRS (~15%)	Variant 3a/b	"Borderline case"
11	Pos.	SS, SRS (~40%)	Variant 1	
12	Neg.	Neg. (<3%)	Neg.	
13	Neg.	Neg. (<3%)	Neg.	
14	Pos.	Mainly SS (~74%)	Exon 6 (EML4) to Exon 19 (ALK)	New Variant[23]
15	Pos.	Mainly SRS (~70%)	Variant 3a/b	CL-H2228

FISH, fluorescence in-situ hybridization; IHC, immunohistochemistry; Pos., positive; Neg., negative; SRS, single red signal; SS, split signal; SGS, single green signal; ADC, adenocarcinoma; SCC, squamous cell carcinoma; CL, cell line.

obtained eight cases (24 cores), TMA-2 the other seven cases (21 cores). Two landmark cores (palatine tonsils) serving as orientation and as a negative control were placed in the right upper corner of each TMA. Each TMA was cut in 43 serial sections, each of 3- to 4-µm thickness. TMA-based ALK-FISH was performed at Berlin (sections 1, 22, and 42) and Heidelberg (sections 2, 24, and 43). This re-evaluation confirmed the above-described ALK-status (on whole slide tissue section) in the first, middle, and last sections of the TMAs (Fig. 1).

Harmonization of the Staining Instruments and Observer Training

Before the multicenter TMA-based case testing, each staining instrument (14× VENTANA BenchMark XT, 2× BenchMark GX [Tucson, AZ]) was calibrated and qualified using the VENTANA ALK 2 in 1 Control Slides. Each participant performed at least four control runs with acceptable staining patterns according to the VENTANA ALK-Interpretation-Guide.[25] To provide a uniform baseline interpretation, a webinar-based training was given to all observers. This training included a virtual meeting to review the VENTANA ALK-Interpretation-Guide and two Training Sets of ten virtual scanned (VENTANA Image Viewer) cases (H&E and IHC). Subsequently each participant had to perform a review of another 50 patient cases (H&E and IHC, all FISH-confirmed), as described elsewhere.[24] All participants passed this ALK-proficiency exam as they performed ALK classifications correctly in at least 45 cases (90%).

Panel-Test Workflow

TMA slides 5, 23, and 39 were H&E-stained and made available electronically as digital slides (Nanozoomer 2.0; Hamamatsu) for reviewing by all participating institutes (Fig. 1). Evaluation included the determination of acceptability of every single core concerning the amount of tumor cells. A mean of 43.3 of 45 H&E cores (96.3%; ratio: 42–45 cores) was accepted by the participants and demonstrated the quality of the TMAs at the different levels. The other slides were shipped to the participating institutes of pathology (Belgium, Denmark, France, Germany, Scotland, Spain, Sweden, and Switzerland) within 7 days after sectioning. Each participant received four slides (two per TMA). After each institute had performed the single IHC-staining steps (for staining procedure see below), every observer evaluated the tissue quality (sufficient tumor tissue, extent of necrosis) of the four obtained TMA slides. A total of 703 of 720 cores (97.6%) were scored suitable (ratio: 42–45 cores per participant). Thus, at least two out of three cores per case could be scored.

Each observer performed independent testing without knowledge of the FISH results. The overall ALK-IHC status of every case had to be reported as negative or positive (binary interpretation) using the ALK (D5F3) Rabbit Monoclonal Primary Antibody combined with the OptiView DAB IHC detection and the OptiView Amplification kits (all VENTANA). The ALK status was called positive if tumor cells in at least one of the three cores of a case showed a strong cytoplasmic staining pattern.[25] A case was called ALK negative if all three cores showed no adequate staining pattern according to the ALK Interpretation Guide.[25]

All results including the stained TMAs were sent to the Institute of Pathology Charité, Berlin for comparison and data collection. All slides were scanned (Nanozoomer 2.0; Hamamatsu, Hamamatsu City, Japan) and included in a PDF forming the basis for a consecutive telephone conference (Supplemental Digital Content, http://links.lww.com/JTO/A689).

As the overall concordance of the results was high, a special focus was set on case 8 and 11 which was not called clearly ALK-IHC positive by a total of five observers. A

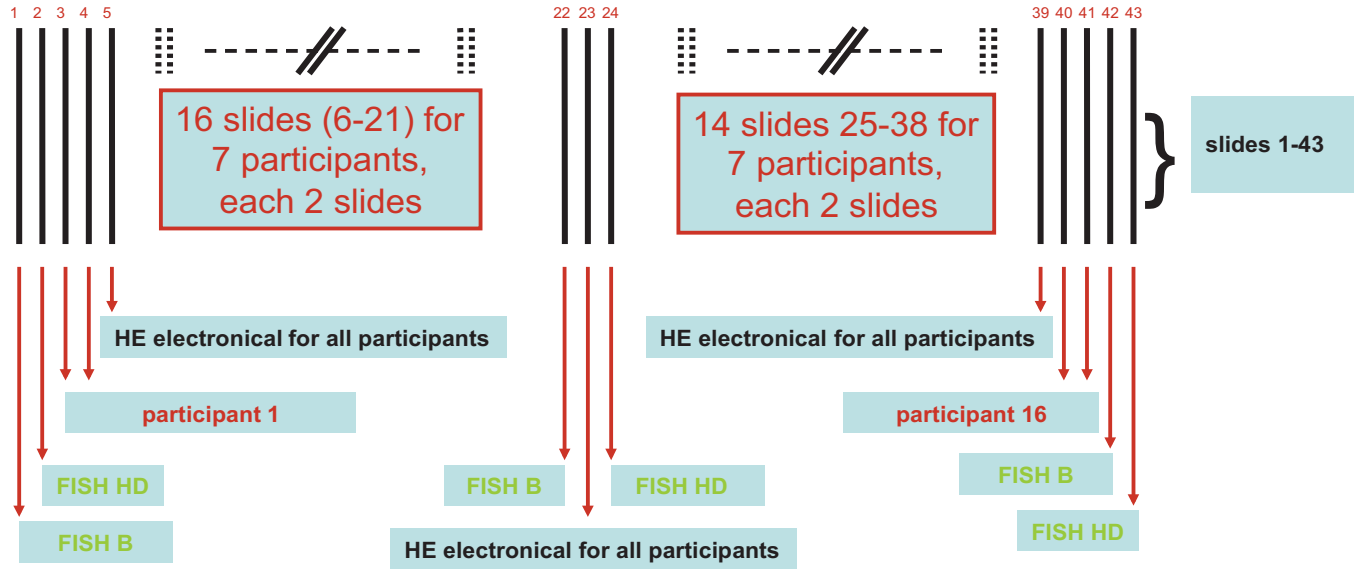

FIGURE 1. Preparation and testing modality of the tissue microarrays (TMA): each of the two TMAs was cut into 43 slides. Slides 1, 2, 22, 24, 42, and 43 were tested by means of ALK-FISH in Berlin (B) and Heidelberg (HD). Slides 5, 23, and 39 were stained by H&E and were electronically available as scans.

strong variance was seen within two observers (institutes 5 and 8). Therefore the following question rose: *Is the ALK-IHC-staining intensity dependent of the time interval between TMA-cutting and staining?*

To address this issue the following steps were conducted:

1. Institutes 5 and 8 performed a restaining of further TMA sections.
2. All staining results were correlated with the exact time interval (days from cutting to staining).
3. An additional TMA staining was performed after 128 days of being cut (at Berlin, Charité).

ALK-D5F3 IHC

The VENTANA anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody has been developed for use on VENTANA BenchMark XT and BenchMark GX automated slide stainers in combination with Rabbit Monoclonal Negative Control Ig, OptiView DAB IHC detection and OptiView Amplification kits. All 16 laboratories performed the staining procedure according to the manufacturer's instructions and as described elsewhere.[26] As an additional component, staining of the 2 in 1 control slide was performed at each procedure. This slide contains two human cell lines (ALK-positive and ALK-negative) serving as controls for the staining quality. An adequate staining procedure with the D5F3-clone can be assumed when one cell line is strongly positive and the other negative.

For adequate interpretation a special step-by-step procedure was recommended: (1) the observers had to review the virtual H&E slides, to determine the amount of tumor cells and the number of adequate cores per case (x/3). (2) The adequacy of the staining procedure had to be evaluated by interpreting the 2 in 1 control slides. (3) The staining quality and intensity of the Negative Control Slide and the ALK-D5F3 tissue slide had to be compared to determine false positive (e.g., macrophages, nerve fibers, and necrosis) and specific tumor staining patterns (strong cytoplasmic).

RESULTS

Binary ALK-IHC Evaluation of 15 NSCLC Samples

Despite some artifacts as staining of macrophages, nerve fibers, and stroma components all 16 participants designated all seven ALK-FISH-negative cases as ALK-IHC-negative (Table 2, Fig. 2). All observers scored the two ALK-FISH positive-"borderline" cases (RT-PCR: EML4-ALK variant 1 and 3a/b) as positive according to their ALK expression profile. This concordant IHC interpretation was also noticed in four of the six unequivocally ALK-FISH positive samples. In the remaining two cases (number 8 and 11), a weak and heterogeneous staining was described by five observers. All of them performed staining on the BenchMark XT platform (Tables 2 and 3). This equivocal pattern would have resulted in a subsequent testing by FISH (and/or PCR) under diagnostic conditions (Table 3, Fig. 2).

In detail, one participant called case 8 ALK negative, according to the strict rules of the ALK Interpretation Guide,[25] as a strong cytoplasmic staining pattern was missing. However, the observer stated the occurrence of a weak and heterogeneous staining pattern, which would have resulted in additional ALK-FISH-testing in daily routine practice. The same was true for case 11. Four participants scored this case negative according to the ALK Interpretation Guide.[25] Still, all of them described a heterogeneous, focally weak and "stippled" staining pattern and would have demanded (as well as three other participants) for an additional independent ALK-test (FISH and/or PCR).

TABLE 2. IHC Testing of Seven ALK-Negative Samples by 16 Institutes

Participant (BM)	Case 2	Case 4	Case 5	Case 7	Case 9	Case 12	Case 13
1 (XT)							
2 (XT)							
3 (GX)							
4 (XT)							
5 (XT)							
6 (XT)							
7 (XT)							
8 (XT)							
9 (XT)							
10 (XT)							
11 (XT)							
12 (XT)							
13 (GX)							
14 (XT)							
15 (XT)							
16 (XT)							

Positive ALK-status in yellow.
BM, BenchMark GX or XT.

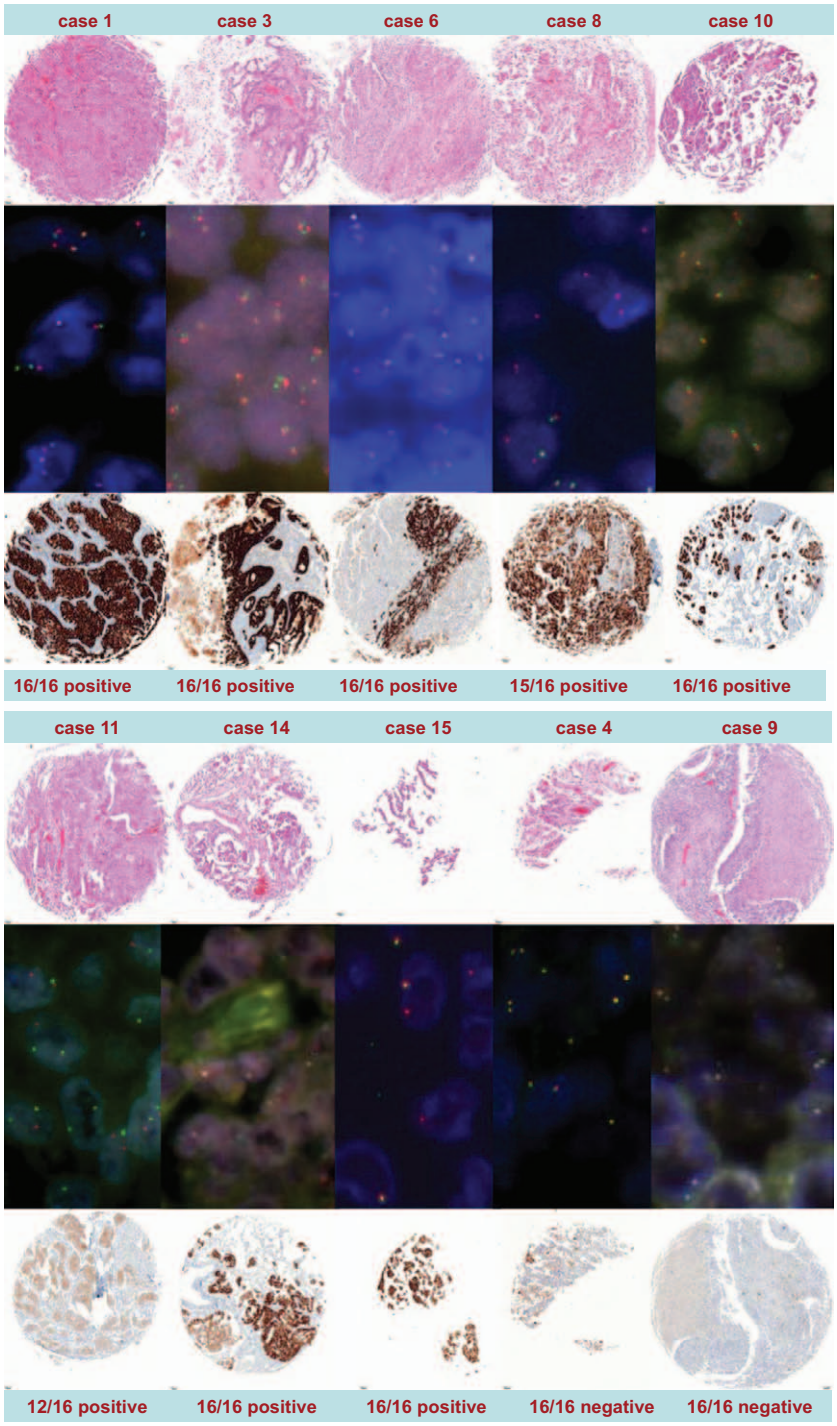

FIGURE 2. Ten NSCLC cases stained with H&E, ALK-IHC (10× objective), and FISH (63× objective). Two ALK-negative cases with challenging IHC due to positivity of macrophages and necrosis are shown in cases 4 and 9. The latter displays a single green signal pattern (SGS) in FISH. At the bottom the interpretation of all 16 observers (negative versus positive) is given. Note the heterogeneous staining pattern in case 11.

Comparison of the Staining Intensities of all Panellists and Restaining

The comparison of the staining intensities showed minor differences between the majority of observers. However, major differences between two observers (institutes 5 and 8) especially in case 8 and 11 (Table 3). IHC staining by all participants was performed between days 5 and 38 after TMA cutting. The weak staining of institutes 5 and 8 referred to days 23 and 36. Nevertheless a very strong cytoplasmic staining pattern was seen for example at days 24 and 38 in other institutes (see Supplemental Digital Content, http://links.lww.com/JTO/A689). The restaining of institutes 5 and 8 showed much stronger staining patterns compared to their first run and was in line with the staining intensities of the other participants. The IHC run performed after 128 days showed strongly reduced IHC intensity: two of eight ALK-positive cases could not be detected as unequivocally positive any more (Supplemental Digital Content, http://links.lww.com/JTO/A689).

TABLE 3. IHC-Testing of Eight ALK-Positive Samples by 16 Institutes

Participant (BM)	Case 1	Case 3	Case 6 (BL*)	Case 8	Case 10 (BL*)	Case 11	Case 14	Case 15
1 (XT)				FISH		FISH		
2 (XT)								
3 (GX)								
4 (XT)								
5 (XT)						FISH**		
6 (XT)						FISH/PCR		
7 (XT)								
8 (XT)				FISH**				
9 (XT)						FISH**		
10 (XT)								
11 (XT)								
12 (XT)						FISH**		
13 (GX)								
14 (XT)								
15 (XT)						FISH**		
16 (XT)						FISH		

Both ALK-FISH-"borderline" cases (=*BL) had been detected by all participants, in case 8, one observer and in case 11, four observers called staining as equivocal/low level staining/questionable negative and of uncertain significance (**). They stated that they would have tested with add on ALK-FISH. In these two cases, another three observers would have tested with add on FISH and/or PCR despite of their positive IHC-result (negative ALK-status in red, positive ALK-status in yellow).

BM, BenchMark GX or XT.

DISCUSSION

The main methods of ALK testing are IHC and FISH. The latter is a validated FDA approved test (companion diagnostic). However, FISH has some technical and interpretational disadvantages. Furthermore, as approximately 80 to 90%[27–29] of lung cancer diagnosis is based on small biopsies, the amount of tumor cells can be too low to perform a dependable ALK-FISH diagnosis. Therefore, to improve the quality of ALK testing, several studies compared IHC and FISH with encouraging results. However, due to the occurrence of ALK IHC-positive/FISH-negative samples (and vice versa!), these studies had not been sufficient to establish IHC as the standalone diagnostic approach.[15–21] Furthermore, it must be noted that a majority of the cited investigations was validated and performed within single laboratories, whereas multicenter use of ALK-IHC showed heterogeneous results according to different antibodies, dilutions, detection, and amplification systems obtained.[12]

In this study, we investigated and proved the multicenter reliability of an IHC-based ALK testing. The basis for multicenter applicability was the harmonization of the staining protocol within the staining instruments, a preceding validation procedure of each instrument and a webinar-based teaching of all participants. Teaching is highly recommended[24] as the described ALK-detection-approach might cause positivity not only in tumor cells, but also in other tissue components such as macrophages, nerve fibers, and areas of necrosis (see cases 4 and 9, Fig. 2*B*). Homogeneous (ALK-protein negative) results were shown in all seven NSCLC-samples without ALK rearrangement. The same was true for six of eight ALK-rearranged samples. Thereby, it is of special interest that all 16 participants scored both ALK-"borderline" samples positive according to their ALK protein expression. ALK-testing of these "borderline" samples by 16 different observers based on FISH only would have led to very deviating ALK-status interpretations (misinterpretations!). However, both samples were RT-PCR confirmed EML4-ALK variants and are treatable targets that are mandatory to detect! One of the two was part of a recent retrospective study,[12] where we could show that three of eight experienced observers did not evaluate this sample as ALK-positive due to technical limitations of the FISH-approach, especially in samples harboring subtle SS patterns.[12,13] The here described two "borderline" samples might quasi represent these kind of NSCLC reported elsewhere as IHC positive and FISH negative, but sensitive to an ALK-TKI.[19,20,30] As the reduction of tumor volume and the response to ALK-inhibitors in ALK-positive-NSCLC was shown to be independent of the amount of ALK-positive tumor cells,[31] the detection of cases with subtle splits (around the cut-off of 15%) is of high importance. Therefore, in these samples a predictive ALK-interpretation cannot be based upon FISH only.

In two unequivocally ALK-break positive samples a few observers described a weaker and heterogeneous "stippled" IHC-staining pattern (Table 3). Even though the participants felt to interpret this equivocal ALK pattern as negative due to the strict rules of the ALK Interpretation Guide,[25] all wished to perform an add-on FISH and/or PCR test. Interestingly all observers reporting an equivocal result performed staining on the BenchMark XT platform, whereas the two investigators referring to the GX did not describe these kind of weak expression patterns. Regardless, this phenomenon was found in two cases only. Furthermore, all remaining participants using the XT did not report questionable IHC patterns (Table 3). Thus, this effect is unlikely a matter of platform, but should be more likely discussed as being due to tumor heterogeneity or (even more plausible) as related to tissue damage of the TMA spots

and technical (platform independent) issues. The latter argument is underlined by the fact that restaining showed stronger staining patterns compared to their first run and was in line with the staining intensities of the other participants. Regardless, our results showed that slight differences in the intensities of staining might occur even if all efforts for harmonization of all components are done (Supplemental Digital Content, http://links.lww.com/JTO/A689). The latter might be due to local laboratory reasons (e.g., air pressure, temperature, and water quality) and seems to be independent of the time interval between cutting and staining (if performed within the first 38 days). Staining results after 128 days showed that IHC intensity was strongly reduced and that two of eight ALK-positive cases could not be detected as unequivocally positive any more (Supplemental Digital Content, http://links.lww.com/JTO/A689).

One major issue of future ALK interpretation will be to identify the frequency of samples with a deviating IHC/FISH ALK-status. As discussed above IHC-positive/FISH negative samples that are sensitive[30] to ALK-inhibitor therapy might be "borderline" cases with very subtle ALK-breaks that might not be detected adequately by FISH.[19] Thus, in these cases a validated ALK-IHC assay might serve as a stand-alone test if the staining pattern is unequivocal. However, this needs to be proven in future clinical trials. On the other hand, the rather rudimentary knowledge of IHC-negative/FISH-positive NSCLC needs further investigation as well[19]:

- Is it a technical reason with false negativity of IHC or false positivity of FISH?
- Is it biology with a genomic alteration within the ALK-gene that does not lead to a cytoplasmic accumulation of the ALK-protein?
- Do these samples show a specific FISH pattern (e.g., single red signals only)?
- What about the therapy response data?

Future studies combining these molecular and clinical key questions of ALK discrepancy could help to implement reliable test algorithms. These could be mainly based on the detection of the ALK protein (prescreening) with FISH being required only if IHC is in doubt (Fig. 3). This would not only clarify and specify ALK reporting but also could help to save time, money, and tissue for additional molecular tests. Furthermore, it would identify exactly those patients who benefit of the TKI. Meanwhile the presented ALK-IHC approach seems to be an alternative predictive option in samples with reduced FISH interpretability (e.g., minimal tumor content in small biopsies, decalcified or altered tissue, and subtle splits).

To conclude, after harmonization of the staining instruments and training of the observers, the ALK-D5F3 IHC

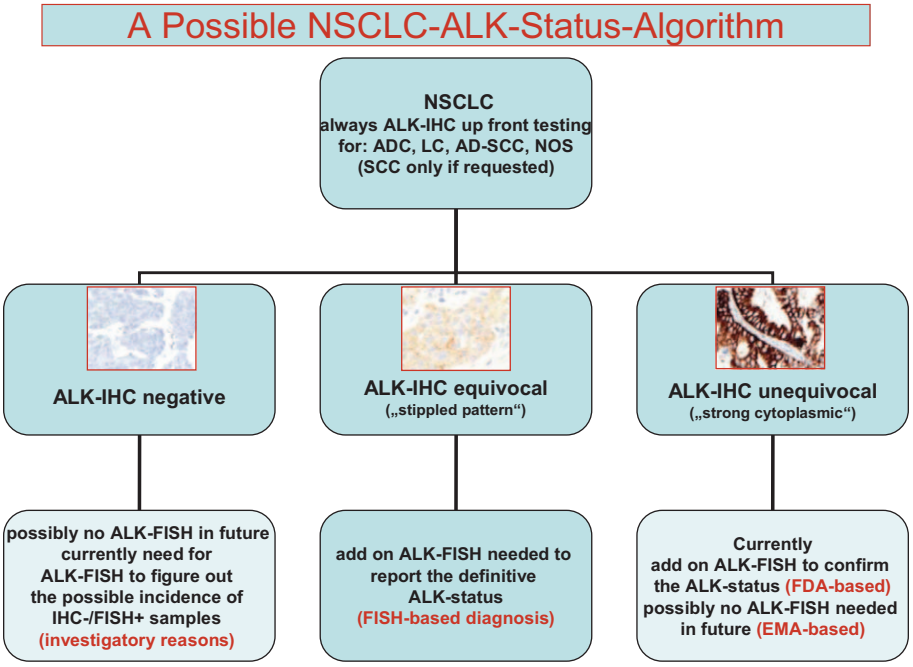

FIGURE 3. ALK-IHC-testing-proposal: a reliable IHC approach needs to be embedded in upcoming algorithms of NSCLC ALK-status testing. ALK-IHC may deliver three results: (a) ALK-IHC negative: in future, no additional ALK-FISH testing may be required (EMA-based concept). Therefore, upcoming clinical trials need to evaluate incidence and therapy-response data in patients with IHC-/FISH+ tumors. At the moment ALK-FISH should be performed if TKI-therapy is an option (FDA-based concept). (b) ALK-IHC equivocal ("stippled" staining pattern): at the moment and in future add-on ALK-FISH should be performed to define the final ALK-status. Therapy decision is based upon FISH (FDA-based concept). (c) ALK-IHC unequivocal (positive): in future, no additional ALK-FISH testing may be required (EMA-based concept). Therefore, upcoming clinical trials need to evaluate incidence and therapy-response data in patients with IHC+/FISH- tumors. It will be of highest interest if those samples show false negativity in FISH due to technical reasons and if the fusion transcript can be detected by PCR. At the moment ALK-FISH should be performed even if ALK-IHC is unequivocally positive (FDA-based concept).

assay in combination with OptiView DAB IHC detection and OptiView Amplification kits can be regarded as a reliable multicenter technique for the detection of ALK protein expression. However, there is need to compare validated ALK IHC assays and ALK-FISH in future clinical trials. Therapy response data of patients with deviating ALK status by means of IHC and FISH will help to implement ALK-IHC not only as a prescreening tool, but also as a potential stand-alone test (at least in cases displaying an unequivocally staining pattern) and as the dependable alternative predictive test in samples with reduced FISH interpretability. Until then, as FISH has some disadvantages and even validated IHC may produce equivocal staining patterns, currently ALK diagnosis should be based on the rational application of both methods (Fig. 3) adapted to the given case.

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